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## PI3K/PTEN/AKT PATHWAY

*A critical mediator of oncogenic signaling*

**Abstract.** Phosphoinositide 3-kinase (PI3K) signaling plays a pivotal role in translating the detection of extracellular cues into alterations in a variety of cellular functions. During states of cellular quiescence this pathway is likewise quiescent. However, in human cancers deregulation of this pathway, typically manifested by constitutive activation, is a common occurrence. In this chapter, the salient properties of this pathway as they relate to cancer development and progression are reviewed. In addition, emerging opportunities for its pharmacological manipulation in the treatment of cancer are discussed.

### 1. INTRODUCTION

Phosphoinositide 3-kinase (PI3K) plays a crucial role in effecting alterations in a broad range of cellular functions in response to extracellular signals. A key downstream effector of PI3K is the serine-threonine kinase Akt which in response to PI3K activation, phosphorylates and regulates the activity of a number of targets including kinases, transcription factors and other regulatory molecules. A causal link between activation of PI3K and the process of cellular transformation was first appreciated in the mid 1980's when the oncogenic activity of Middle T antigen of Polyoma virus was linked to its ability to induce PI3K activity. A major role for PI3K pathway activation in human tumors has been more recently established following both the positional cloning of the *PTEN* tumor suppressor gene, and the discovery that the PTEN protein product was a lipid phosphatase that antagonizes PI3K function and consequently inhibits downstream signaling through Akt.

Subsequently a number of the components of the pathway have been found mutated or deregulated in a wide variety of human cancers highlighting the key role of this pathway in cellular transformation.

A comprehensive review of the PI3K/PTEN/Akt pathway is beyond the scope of this chapter and has been covered elsewhere (Fruman, Meyers, & Cantley, 1998; Katso et al., 2001; Vanhaesebroeck & Waterfield, 1999).

### 2. THE PATHWAY

#### 2.1. Overview

Phosphoinositides (PtdIns) are rare lipids. A large family of lipid kinases are capable of phosphorylating these lipids and are sub-classified based upon their structure and preferred substrates. The class I PI3Ks catalyse the conversion of phosphatidylinositol-3,4-bisphosphate (PtdIns-4,5-P<sub>2</sub>) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P<sub>3</sub>). PtdIns-3,4,5-P<sub>3</sub> is absent or undetectable in resting

cells but is acutely increased in response to multiple stimuli that activate type I PI3K.

A large number of the plasma membrane receptors, in particular those with tyrosine kinase activity, can activate class I PI3Ks. For instance, binding of insulin-like growth factor-1 (IGF-1) to its cognate receptor IGF1R leads to receptor activation and autophosphorylation on tyrosine residues. This in turn leads, through an adaptor molecule, to the recruitment of PI3K to the membrane. Cytokines and cell attachment to the extracellular matrix also stimulate PI3K activity. Once activated and localized to the membrane, PI3K phosphorylates phosphoinositol lipids on the D3 position of the inositol ring generating PtdIns-3-phosphates (PtdIns-3,4-P<sub>2</sub> and PtdIns-3,4,5-P<sub>3</sub>). These specialized lipids serve to recruit pleckstrin homology (PH) domain-containing proteins such as the serine-threonine kinase Akt and PDK1 (phosphoinositide-dependent kinase 1) to the plasma membrane. After recruitment to the membrane, Akt is phosphorylated and consequently activated, by PDK. In turn, Akt phosphorylates multiple proteins on serine and threonine residues (see Figure 1 and further below). Through phosphorylation of these targets, Akt carries out its role as a key regulator of a variety of critical cell functions including glucose metabolism, cell proliferation and survival.

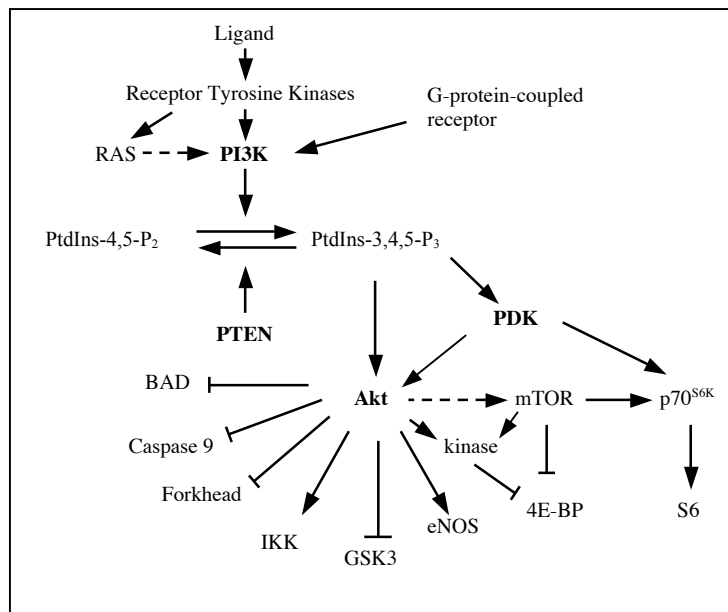


Figure 1. The PI3K/PTEN/Akt pathway. GSK3 (glycogen synthase 3), p70<sup>S6K</sup> (ribosomal protein S6 kinase), BAD (Bcl-2, Bcl<sub>xL</sub>-antagonist causing cell death), IKK (I $\kappa$ B kinase), eNOS (endothelial nitric oxide synthase), mTOR (mammalian target of rapamycin) 4E-BP (eukaryotic translation initiation factor 4E binding protein).

This pathway is highly conserved among different species including *Drosophila melanogaster*, *Caenorhabditis elegans* and mammals. Studies in *Drosophila* have established the involvement of this pathway in the regulation of cell size and number (Brogiolo et al., 2001; Goberdhan, Paricio, Goodman, Mlodzik, & Wilson, 1999; Huang et al., 1999; Oldham, Bohni, Stocker, Brogiolo, & Hafen, 2000; Verdu, Buratovich, Wilder, & Birnbaum, 1999). Genetic studies in *C. elegans* have linked this pathway to regulation of the dauer formation. The dauer phenotype is a larval state characterised by developmental arrest and reduced metabolic rate triggered by adverse environmental conditions including nutrient deprivation and overcrowding. Genetic dissection of the genes involved in this pathway led to the identification of the *daf* (dauer affected) genes (Lin, Dorman, Rodan, & Kenyon, 1997; Ogg & Ruvkun, 1998), some of which are the homologs of the mammalian components of the insulin-PI3K signaling. Figure 2 depicts the main components of this pathway conserved among different species.

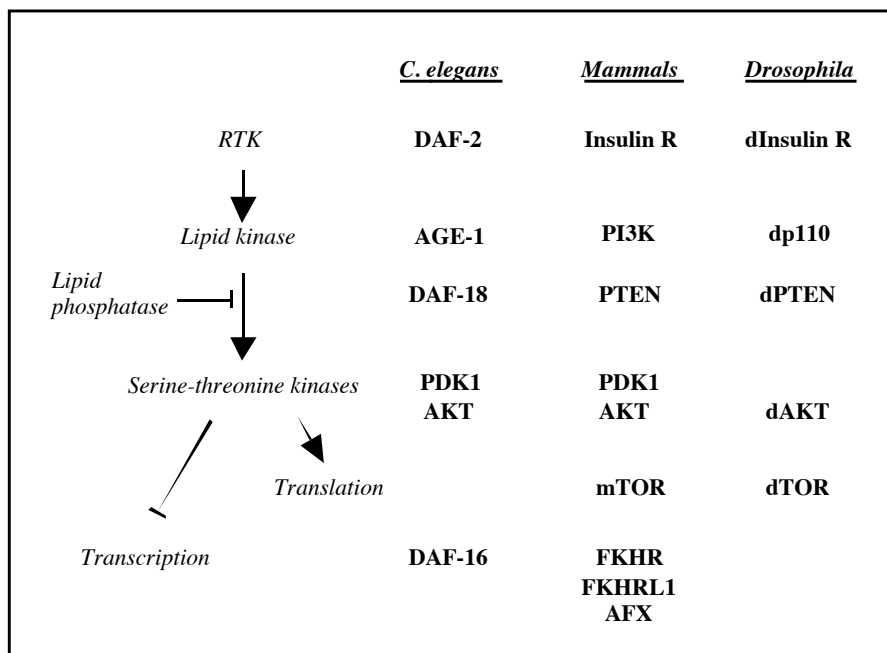


Figure 2. The PI3K/PTEN/Akt pathway in different species.

## 2.2. Phosphoinositide 3- kinases (PI3K).

PI3K belongs to a large family of PI3K-related kinases or PIKK. Other members of the family include mTOR (mammalian target of rapamycin), ATM (ataxia-telangiectasia mutated), ATR (ATM and RAD3 related), DNA-PK (DNA-dependent

protein kinase). All possess the characteristic PI3K-homologous kinase domain and a highly conserved carboxyl-terminal tail (Kuruville & Schreiber, 1999). However, only PI3K is known to have an endogenous lipid substrate. Importantly, all members of the PIKK family have been implicated in human cancer both as oncogenes in the case of type I PI3K or as tumor suppressor genes in the case of ATM and ATR.

The PI3K family (see table 1) comprises eight members divided into three classes according to their sequence homology and substrate preference (reviewed in (Fruman et al., 1998; Vanhaesebroeck & Waterfield, 1999). All mammalian cells express representatives of the three groups. The first member of the family was isolated in 1990 (Carpenter et al., 1990).

Class I: four members have been identified and are further subclassified on the basis of their mechanism of activation. Class Ia, including p110 $\alpha$ , p110 $\beta$  and p110 $\delta$ , associate with a p85 regulatory subunit to form a heterodimeric complex. There are 8 isoforms of p85 encoded by three genes, each containing two SH2 (Src homology) domains that interact with phosphotyrosines on activated RTKs. This results in recruitment of the enzyme to the plasma membrane and activation of the enzymatic activity. For instance, both PDGFR (platelet-derived growth factor receptor) and IR (insulin receptor) have binding sites for p85 and thus strongly activate class Ia PI3K upon binding to their cognate ligands. In addition, activated (GTP-bound) RAS can activate class Ia kinases by direct interaction with the catalytic subunit (Downward, 1998).

Table 1. PI3K family members

Class	Catalytic subunit	Regulatory subunit	Activation	Products
Ia	p110 $\alpha$ p110 $\beta$ p110 $\delta$	p85	RTK, RAS	PtdIns-3,4,5-P <sub>3</sub> PtdIns-3,4-P <sub>2</sub> PtdIns-3-P
Ib	P110 $\gamma$	p101	Heterotrimeric G proteins	PtdIns-3,4,5-P <sub>3</sub> PtdIns-3,4-P <sub>2</sub> PtdIns-3-P
II	PI3KC2 $\alpha$ PI3KC2 $\beta$ PI3KC2 $\gamma$		RTK, integrins	PtdIns-3,4,-P <sub>2</sub> PtdIns-3-P
III	VSP34p			PtdIns-3-P

Genetic studies in the mice have highlighted the role of p110 $\alpha$  in regulating cell proliferation during embryo development. Indeed, homozygous deletion of the gene encoding p110 $\alpha$  (*Pik3ca*) in the mice is embryonic lethal due to a complete lack of proliferation at embryonic day 9.5 (Bi, Okabe, Bernard, Wynshaw-Boris, & Nussbaum, 1999). Interestingly, the p85 regulatory subunit was highly

overexpressed in these mice. Thus, a dominant negative effect on the remaining p110 $\alpha$  and p110 $\beta$  cannot be ruled out.

Class Ib has one member, p110 $\delta$ , which is activated by  $\beta\gamma$  subunits of the heterotrimeric G proteins, which are released upon activation of seven transmembrane receptors. p110 $\delta$  is expressed primarily in leukocytes.

Class II comprises three members (PI3KC2 $\alpha$ ,  $\beta$  and  $\gamma$ ) characterised by a carboxyl-terminal phospholipid-binding domain. While no regulatory subunit has been identified, class II enzymes are predominantly membrane bound and activated by membrane receptors including RTKs and integrins.

The Class III kinase VPS34p is responsible for producing the majority of the cellular PtdIns-3-P and is involved in protein trafficking through the lysosome.

### 2.3. The tumor suppressor PTEN

PTEN (phosphatase and tensin homolog deleted on chromosome 10)/MMAC1 (mutated in multiple advanced cancers)/TEP-1(TGF $\beta$ -regulated and epithelial cell-enriched phosphatase) (hereafter referred to as PTEN) is a tumor suppressor gene localized to chromosome 10q23 (Li & Sun, 1997; Li et al., 1997; Steck et al., 1997). The PTEN protein is both a protein and a lipid phosphatase (reviewed in (Cantley & Neel, 1999; Maehama, Taylor, & Dixon, 2001; Vazquez & Sellers, 2000). The phosphatase domain has homology to protein tyrosine phosphatases, dual-specificity phosphatases, and to tensin and auxilin (Li & Sun, 1997; Li et al., 1997; Steck et al., 1997). The lipid phosphatase activity of PTEN can dephosphorylate the D3 position of PtdIns-3,4-P<sub>2</sub> and PtdIns-3,4,5-P<sub>3</sub>, the lipid products of the PI3K lipid kinase activity (Maehama & Dixon, 1998). Thus, PTEN antagonizes signaling through the PI3K pathway. Indeed, cells lacking PTEN function exhibit a two fold increase in PtdIns-3,4,5-P<sub>3</sub> levels (Stambolic et al., 1998; Sun et al., 1999).

PTEN can also dephosphorylate tyrosine-, serine-, and threonine-phosphorylated peptides (Myers & Tonks, 1997). This activity may be related to regulation of cell adhesion and spreading. When overexpressed in cells, PTEN can dephosphorylate focal adhesion kinase (FAK) (Tamura et al., 1999) and the adaptor protein Shc (Gu et al., 1999). In addition, expression of PTEN results in a decrease in cell spreading and motility (Tamura et al., 1998). However, the relevance of the protein phosphatase activity for PTEN tumor suppression is unclear as certain tumor- and germline-derived mutants of PTEN give rise to protein products that retain their protein phosphatase activity (Furnari, Huang, & Cavenee, 1998; Myers et al., 1998; Ramaswamy et al., 1999). These findings suggest that this activity is not sufficient to block tumor development. Indeed, the preponderance of the published data suggests that PTEN's role as a tumor suppressor is mediated largely through its lipid phosphatase activity.

Analysis of PTEN crystal structure shows that in addition to the catalytic domain PTEN has a C2 domain (Lee et al., 1999). The C2 domain binds lipids and thus may serve to position the catalytic domain at the plasma membrane. PTEN also has a C-terminal "tail" that contains a PDZ domain. PDZ domains are protein-protein interaction modules that play a critical role in organizing diverse cell signaling

complexes. Phosphorylation of three residues (S380, T382, and T383) within the tail is necessary for maintaining protein stability and also acts inhibiting PTEN function (Adey et al., 2000; Georgescu et al., 2000; Tolkacheva et al., 2001; Vazquez, Ramaswamy, Nakamura, & Sellers, 2000). Phosphorylation of the tail acts as an inhibitory switch. When phosphorylated, PTEN is in a “close”, monomeric conformation with low affinity for PDZ-domain containing proteins. Conversely, the unphosphorylated form is in an “open” conformation that allows recruitment to high molecular weight complexes (Vazquez et al., 2001). These complexes comprise PDZ-domain containing proteins, such as MAGI-2, and they are thought to be important for PTEN localization to the plasma membrane (Vazquez et al., 2001; Wu et al., 2000). Once localised to the membrane, PTEN can exert its phospholipid phosphatase activity.

Targeted disruption of *Pten* in the mice leads to embryonic lethality (Di Cristofano, Pesce, Cordon-Cardo, & Pandolfi, 1998; Podsypanina et al., 1999; Stambolic et al., 2000; Sun et al., 1999; Suzuki et al., 1998). Abnormal proliferation but not significant differences in apoptosis are observed in the mutant embryos. Interestingly, *Pten*<sup>+/-</sup> mice, as the Cowden’s syndrome patients (see further below), are cancer prone and develop a range of neoplasms including tumors of the breast, endometrium prostate, liver, gastrointestinal tract, thyroid and thymus and T-cell lymphomas (Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 2000; Suzuki et al., 1998). The majority of these tumors exhibit loss of the wild type allele, underscoring the importance of loss of PTEN function in tumor formation.

In the fruit fly *Drosophila melanogaster* loss of PTEN function is lethal in the larval stage. Importantly, this lethality can be rescued by a PH domain mutant Akt (Stocker et al., 2002). This Akt mutant has a reduced affinity for PtdIns-3,4,5-P<sub>3</sub> suggesting that PH-domain mediated activation of Akt is the only lethal event triggered by increased levels of PtdIns-3,4,5-P<sub>3</sub> in PTEN-null cells. Thus, *D. melanogaster* Akt appears to be the most critical effector downstream PTEN.

#### 2.4. The serine/threonine protein kinase Akt

The serine-threonine protein kinase Akt (also known as protein kinase B, PKB) mediates many of the downstream effects of PI3K and consequently plays a central role in both normal and pathological signaling by the PI3K pathway.

There are three closely related enzymatic isoforms Akt1 (PKB $\alpha$ ), Akt2 (PKB $\beta$ ) and Akt3 (PKB $\gamma$ ) encoded by three different genes located on chromosomes 14q32, 19q13 and 1q43 respectively. They are similar both in structure and size and are thought to be activated by a common mechanism (Okano, Gaslightwala, Birnbaum, Rustgi, & Nakagawa, 2000). To date, no differences in substrate preference have been established are currently assumed to have identical or similar substrate specificity. The three isoforms are widely expressed though Akt3 tissue distribution seems to be more restricted than 1 and 2, being primarily expressed in brain and testis (Konishi et al., 1995).

The three Akt proteins (henceforth referred to as Akt) contain an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal regulatory region. The PH domains of Akt and related kinases such as BTK (Bruton's tyrosine kinase) can bind specifically to D3-phosphorylated phosphoinositides with high affinity.

Activation of Akt is a multi-step process involving both membrane binding and phosphorylation. Upon PI3K activation and production of PtdIns-3,4,5-P<sub>3</sub> and PtdIns-3,4-P<sub>2</sub>, Akt is recruited to the plasma membrane where it binds to these phosphoinositides through its PH domain (Franke, Kaplan, Cantley, & Toker, 1997). Activation is then thought to involve a conformational change and phosphorylation on two residues. One such phosphorylation site lies within the kinase domain activation loop (Thr 308 in Akt1) and is phosphorylated by another PH-domain containing protein, PDK1 (reviewed in {Alessi, 2001 #2014}). This is thought to be the major activating phosphorylation event. In addition, a second phosphorylation site in the C-terminus (Ser 473 in Akt1) is required for full or maximal activity. The identity of the serine 473 kinase is not firmly established and this phosphorylation event may result from PDK1 itself (Balendran et al., 1999), from integrin-linked kinase (Persad et al., 2001) or from Akt autophosphorylation. A detailed description of the structural features and mechanism of activation can be found elsewhere (Coffer, Jin, & Woodgett, 1998; Datta, Brunet, & Greenberg, 1999; Galetic et al., 1999; Kandel & Hay, 1999).

Growth factor stimulation of PI3K activity leads to Akt activation. Conversely, PI3K inhibition (i.e. using chemical inhibitors such as wortmannin or LY294002) and PTEN mediated dephosphorylation of PtdIns-3,4,5-P<sub>3</sub> and PtdIns-3,4-P<sub>2</sub> results in inhibition of Akt. After activation, Akt can phosphorylate a number of substrates both in the cytoplasm and in the nucleus.

Disruption of the genes for murine *akt1* and *akt2* gives rise to viable mice that show phenotypic differences. *Akt1*<sup>-/-</sup> mice are smaller and have shorter life span when exposed to genotoxic stress than wild type littermates. In addition, they exhibit increased spontaneous apoptosis in the testis and thymus (Chen et al., 2001) (Cho, Mu et al., 2001). In contrast, *Akt2*<sup>-/-</sup> mice show insulin resistance and a diabetes mellitus-like syndrome (Cho, Mu et al., 2001). Whether these differences result from differences in substrate preference or tissue regulation is not clear. In addition, the viability and relatively mild phenotypes of these knockout mice raise the possibility that the three Akt isoforms can, compensate for each other with respect to functions that might compromise organismal viability. The generation of compound knock-out animals will address this issue.

### 2.5. Akt targets

Akt phosphorylates a variety of substrates involved in the regulation of key cellular functions including cell growth and survival, glucose metabolism and protein translation. These targets include GSK3, IRS-1 (insulin receptor substrate-1), PDE-3B (phosphodiesterase-3B), BAD, human caspase 9, Forkhead and NF- $\kappa$ B transcription factors, mTOR, eNOS, Raf protein kinase, BRCA1, and p21<sup>Cip1</sup> /WAF1

(Altiok et al., 1999; Datta et al., 1999; Galetic et al., 1999; Montagnani, Chen, Barr, & Quon, 2001; Zhou et al., 2001; Zimmermann & Moelling, 1999).

One common mechanism through which Akt-mediated phosphorylation results in substrate inhibition is through the regulation of subcellular localization by interaction with 14-3-3 proteins (i.e. BAD, forkhead transcription factors). 14-3-3 proteins are cytoplasmic proteins that bind specifically to phosphoproteins and retain them in the cytoplasm (Yaffe et al., 1997) away from their targets. In particular the Akt consensus phosphorylation site is also a consensus 14-3-3 binding site (Yaffe et al., 2001). For example, BAD phosphorylation by Akt inhibits its proapoptotic effects. In the unphosphorylated state, BAD is targeted to the mitochondria where it forms a complex with Bcl-2 or Bcl<sub>XL</sub>, inhibiting their anti-apoptotic activity. Conversely, when phosphorylated, BAD associates with 14-3-3 proteins in the cytoplasm.

FKHR, FKHL1 and AFX transcription factors (henceforth referred to as Forkhead) belong to the winged helix/forkhead transcription factors family characterized by a 100-amino acid, monomeric DNA binding domain (DBD)(reviewed in (Kops & Burgering, 1999; Kops et al., 1999)). These three family members are directly phosphorylated and regulated by Akt. Forkhead transcriptional activity is negatively regulated through Akt-dependent phosphorylation on three conserved residues (Biggs, Meisenhelder, Hunter, Cavenee, & Arden, 1999; Brunet et al., 1999; Kops et al., 1999; Rena, Guo, Cichy, Unterman, & Cohen, 1999). Upon phosphorylation, Forkhead binds to 14-3-3 proteins and remains in the cytoplasm where they are thought to be functionally inactive. Recent data suggests that 14-3-3 acts not merely as a cytoplasmic retention signal, but targets nuclear Forkhead to the nuclear export machinery (Brunet et al., 2002). In cancer cell lines lacking functional PTEN, FKHL1 and FKHR are constitutively phosphorylated by Akt and are hence constitutively cytoplasmic and unable to activate transcription (Nakamura et al., 2000). Moreover, reconstitution of PTEN to those cells restores nuclear localization of FKHR and restores its ability to activate promoter elements. Mutation of the three Akt phosphorylation sites to alanine renders FKHR independent of Akt activation. Consequently, it remains localized in the nucleus and hence constitutively active. Importantly, this constitutively active form of FKHR (FKHR;A3) is able to replace PTEN function in PTEN-null cells. Specifically, in PTEN-null cells that undergo G1 arrest upon PTEN reconstitution (i.e. 786-0 cells), likewise FKHR;A3 induces a G1 arrest (Medema, Kops, Bos, & Burgering, 2000; Nakamura et al., 2000). On the other hand, in PTEN-null cells that undergo apoptosis upon PTEN reconstitution (i.e. LNCaP cells) FKHR;A3 likewise induces apoptosis. Thus, Forkhead is a critical effector of both cell-cycle progression and apoptosis downstream of PTEN (Nakamura et al., 2000). In addition, other Forkhead family members have also been implicated in the induction of apoptosis both through the upregulation of FasL (Brunet et al., 1999) and through the regulation of the pro-apoptotic Bcl-2 interacting mediator (Bim1) (Dijkers, Medema, Lammers, Koenderman, & Coffey, 2000).

Human Caspase-9, a member of the protease family intimately associated with the initiation of apoptosis, is thought to be phosphorylated and inhibited by Akt..

(Cardone et al., 1998). However, the Akt phosphorylation site is not conserved in the Caspase 9 proteins from other mammals making its *in vivo* importance unclear.

In addition to the inhibition of pro-apoptotic factors, Akt can also activate the transcription of anti-apoptotic genes through the activation of the transcription factor NF $\kappa$ B (Kane, Shapiro, Stokoe, & Weiss, 1999; Khwaja, 1999; Ozes et al., 1999; Romashkova & Makarov, 1999). When bound to its inhibitor, termed I $\kappa$ B, NF $\kappa$ B localises to the cytoplasm. Akt associates and activates the I $\kappa$ B kinases (IKKs). Activated IKKs phosphorylate I $\kappa$ B targeting it for degradation by the proteasome. This allows NF $\kappa$ B to translocate to the nucleus and activate transcription of a variety of substrates including anti-apoptotic genes such as the inhibitors of apoptosis (IAP) c-IAP1 and 2.

## 2.6. FRAP/mTOR

The ribosomal protein S6 kinases (S6Ks) and the mammalian target of rapamycin (mTOR, also known as FRAP) have been linked to the PI3K signalling, though the exact mechanism for this connection remains to be clarified (reviewed in (Gingras, Raught, & Sonenberg, 2001). In response to growth factor stimulation, S6Ks are phosphorylated in multiple sites and, in turn, phosphorylate the ribosomal protein S6 leading to increase translation. The kinases upstream S6K seem to include PDK1 and mTOR among others.

In addition to S6K, mTOR has been reported to phosphorylate the eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs) positively modulating the initiation of translation. Hypophosphorylated 4E-BPs bind efficiently to eIF-4E forming an inhibited complex. Upon phosphorylation, 4E-BPs dissociate from the complex allowing eIF-4E to incorporate into the translation initiation machinery. A variety of stimuli can activate mTOR kinase activity including mitogens, amino acid availability and ATP levels (Dennis et al., 2001).

In *D. melanogaster*, mutations in the insulin signalling pathway including loss of PTEN act to alter regulation of cell size and cell number. Genetic analysis of this pathway suggests that these effects arise primarily as a consequence of alterations in the function of the TOR homologue in drosophila (Zhang, Stallock, Ng, Reinhard, & Neufeld, 2000). In keeping with these data, in mammalian and avian cells tumors and cancer cell lines harbouring either alterations in PTEN or bearing activated alleles of Akt and PI3K, appear to be exquisitely sensitive to treatment with either rapamycin or the rapamycin analog CCI-779 (Aoki, Blazek, & Vogt, 2001; Neshat et al., 2001; Podsypanina et al., 2001).

## 2.7 Other PI3K effectors

Other downstream effectors include the small GTPase Rac (Kotani, Hara, Yonezawa, & Kasuga, 1995; Posern, Saffrich, Ansorge, & Feller, 2000) and the serine-threonine kinase ILK (integrin-linked kinases) (Dedhar, Williams, & Hannigan, 1999).

### 3. PERTURBATIONS IN CANCER

Carcinogenesis is a multi-step process involving genetic and epigenetic alterations that together lead to the acquisition of six common features of the transformed cell. Namely, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2000). In different ways and degrees, the various components of the PI3K/PTEN/Akt pathway have been related to most of those cellular phenotypes.

#### 3.1. PI3K

As mentioned above, most protein tyrosine kinases (both membrane receptors and cytoplasmic) can signal through the PI3K pathway and oncogenic activation of tyrosine kinases observed in multiple human cancers results in deregulated activation of PI3K activity. Other mechanisms of activation include amplification of the gene encoding the catalytic subunit and activating mutations in the regulatory subunit.

Thus, far there is no direct evidence for activating mutations of PI3K catalytic subunit in human cancers, however *PIK3CA*, the gene coding for p110 $\alpha$ , is frequently amplified (increased in copy number) in ovarian (Shayesteh et al., 1999) and cervical cancers (Ma et al., 2000). The increased copy number is associated with increased *PIK3CA* transcription, p110 $\alpha$  protein expression and PI3K activity, resulting in increase cell growth and decreased apoptosis. Increased enzymatic activity has been also reported in colorectal tumors (Phillips, St Clair, Munday, Thomas, & Mitchell, 1998).

In addition, somatic mutations in the p85 regulatory subunit leading to constitutively activation of the catalytic subunit have been described in ovarian and colon tumors (Philp et al., 2001).

The avian retroviral oncogene v-p3k, a homolog of p110 $\alpha$ , is involved in the induction of hemangiosarcomas in chickens. Here, V-p3k is fused to viral gag proteins resulting in localization to the membrane and, consequently, constitutively enzymatic activation. V-p3k is oncogenic both *in vivo* and *in vitro* (Aoki et al., 2000; Chang et al., 1997). In addition, membrane targeted p110 $\alpha$  induces cell cycle entry and in immortalized rodent cells is sufficient for oncogenic transformation (Klippel et al., 1998).

PI3K is also thought to be implicated in the metastatic phenotype. Indeed, several molecules involved in cell migration and cell adhesion can regulate or be regulated by PI3K. For instance,  $\alpha$ 6 $\beta$ 4 integrins can activate PI3K and promote carcinoma invasion (Shaw, Rabinovitz, Wang, Toker, & Mercurio, 1997). This phenotype appears to be independent of Akt activation. Along this lines, recent studies in transgenic mice suggest an Akt-independent mechanism for PI3K-induced metastatic phenotype (Hutchinson, Jin, Cardiff, Woodgett, & Muller, 2001). PI3K can interact and be activated by E-cadherin (Pece, Chiariello, Murga, & Gutkind, 1999), cell surface molecules involved in cell-cell adhesion. PI3K can also activate the small GTPase Rac (Kotani et al., 1995; Posern et al., 2000) and the serine-

threonine kinase ILK (integrin-linked kinases) (Dedhar et al., 1999). These downstream effectors seem to be required for PI3K-mediated invasion. However, the specific molecular mechanism by which PI3K signalling mediates or induces an invasive phenotype remains under investigation.

### 3.2. *PTEN*

*PTEN* is one of the genes most commonly mutated in human cancers and LOH at the *PTEN* locus on 10q23 is a frequent event in both primary and metastatic tumors. In addition, germ line mutations of *PTEN* are associated with the hereditary cancer predisposition syndromes known as Cowden's disease and related conditions (Liaw et al., 1997). These findings, together with the studies in animal models, strongly support a critical role of *PTEN* in tumor suppression.

#### 3.2.1. *Germline mutations*

Germline mutations in the *PTEN* gene have been reported in two rare autosomal dominant disorders known as Cowden's disease (CD) and Bannayan-Zonana syndrome (BZS). These syndromes are characterized by the development of hamartomas that is lesions characterized by hyperplastic, disorganized and benign tumors. Hamartomas of the outer root sheath of the hair follicle (trichilemmomas) are pathognomonic. In addition, thyroid, breast, skin, and intestinal hamartomas are also found. Loss of the *PTEN* wild type allele in hamartoma tissue is an early event leading to increased proliferation. In addition, CD patients are prone to develop breast and thyroid cancers (Nelen et al., 1997).

Germline *PTEN* mutations giving rise to these syndromes comprise frameshift, nonsense, missense and splice-site mutations typically resulting in the generation of truncated, inactive proteins. Among the missense mutations, a high proportion cluster in the phosphatase domain (Ali, Schriml, & Dean, 1999).

Lhermitte-Duclos disease is a CD associated condition defined by dysplastic gangliocytoma of the cerebellum (Eng et al., 1994). Importantly, it has been recently reported that deletion of *PTEN* in the mouse brain gives rise to lesions that resemble the histopathology of Lhermitte-Duclos cerebellum (Backman et al., 2001; Kwon et al., 2001). Neurons lacking *PTEN* expression exhibit high levels of phosphorylated Akt and show progressive increase in soma size without evidence of abnormal proliferation (Kwon et al., 2001).

#### 3.2.2. *Somatic mutations*

*PTEN* is frequently inactivated in sporadic human tumors from various tissues, including endometrium, brain, prostate, and ovary (reviewed in (Ali et al., 1999; Bonneau & Longy, 2000)). The most common inactivating events are frameshift and missense mutations and homozygous deletions (Ali et al., 1999).

LOH at chromosome 10q has been reported in 60%-80% of glioblastomas (Bostrom et al., 1998; Rasheed et al., 1997; Steck et al., 1999; Wang et al., 1997).

Intragenic mutations in the PTEN gene have been identified in 60% of primary glioblastoma cell lines (Li et al., 1997; Steck et al., 1997) and in approximately 20-40% of glioblastoma multiforme, the most aggressive subtype of astrocytic tumor (Bostrom et al., 1998; Duerr et al., 1998; Li et al., 1997; Liu, James, Frederick, Alderete, & Jenkins, 1997; Rasheed, Wiltshire, Bigner, & Bigner, 1999; Somerville et al., 1998; Steck et al., 1997; Wang et al., 1997). In contrast, in lower grade gliomas (such as anaplastic astrocytomas) although they also harbor chromosome 10q loss mutation of the second PTEN allele is comparatively rare (Bostrom et al., 1998; Duerr et al., 1998). Therefore, PTEN mutation appears to be correlated with higher grade tumors.

In prostate cancer, loss of heterozygosity in the 10q23 interval is found in between 18 and 65% of tumors (reviewed in Ramaswamy S et al., 2000). In addition, *PTEN* promoter methylation and gene silencing has been reported in some prostate cancer xenografts (Whang YE et al., 1998). The PTEN protein is absent in 20% of primary tumor specimens when studied by immunohistochemistry and its absence correlates with advance pathological grade and stage (McMenamin et al., 1999). In addition, as many as 60% of patients with metastatic lesions are found to have a focus of prostate cancer in which PTEN is mutated (Suzuki, 1998 #1457). Thus, *PTEN* loss appears to be a critical step in the development of aggressive and likely lethal forms of prostate cancer.

*PTEN* mutations occur in 30%-50% of endometrial cancers primarily in the endometrioid sub-type (Ali et al., 1999). Similarly, PTEN is mutated in 24% of ovarian cancers again at higher frequency in the endometrioid type (Obata et al., 1998; Yokomizo et al., 1998). In contrast to other tumor types that sustain PTEN mutations, loss of function PTEN mutations are more common in Grade I or early stage tumors of the ovary. Thus, here PTEN may play a role in the tumor initiation (Levine et al., 1998; Maxwell et al., 1998).

PTEN mutations can be found in 30-40% of malignant melanomas (Guldberg et al., 1997; Tsao, Zhang, Benoit, & Haluska, 1998), correlating with a LOH frequency of 30-50% on chr 10q (Healy et al., 1998; Isshiki, Elder, Guerry, & Linnenbach, 1993). In addition, in specific instances, PTEN mutations were found in metastatic foci, but not in the corresponding primary tumors, again suggesting that PTEN is involved in tumor progression (Guldberg et al., 1997).

### 3.3. *Akt*

The first evidence pointing to a role of Akt in tumorigenesis was given by the early studies of the transforming murine virus ATK8 and the finding of *Akt1* and *2* gene amplification in gastric adenocarcinoma (Bellacosa, Testa, Staal, & Tsichlis, 1991; Staal, 1987; Staal & Hartley, 1988). The viral homolog, v-Akt, is a fusion protein containing Gag sequences at its amino terminus (Bellacosa et al., 1991). This fusion creates a myristoylation sequence allowing for a posttranslational modification that directs proteins to the plasma membrane (Ahmed, 1993 #1995).

In human cancer, there are several mechanisms that lead to deregulated Akt activity, inappropriate activation of PI3K, *Akt* gene amplification, Akt protein overexpression and loss of PTEN. Given the high frequency of PTEN mutations in

human cancer, it is likely that the latter mechanism accounts for the majority of Akt activation events.

Akt1 gene amplification has been found in gastric adenocarcinomas (Staal, 1987). In addition, increased Akt1 kinase activity and its association with a poorer prognosis in prostate, ovary and breast carcinomas has been recently described (Sun et al., 2001). In these tumors it is likely that Akt activation resulted from either PTEN loss or PI3K activation rather than direct amplification or activation of Akt (repeat the reference).

Akt2 mutations appear more prevalent or at least, more reproducibly documented in human cancer than Akt 1 or 3. The Akt2 gene is amplified and overexpressed in ovarian carcinomas, both in cell lines and in primary tumors (Cheng et al., 1992); (Bellacosa et al., 1995). Here, amplification was closely associated with an aggressive tumor phenotype. In a recent report, elevated Akt2 kinase activity was found in approximately 40% of primary ovarian cancers (Yuan et al., 2000). Ten percent of pancreatic cancer cells show Akt 2 amplification (Cheng et al., 1996). Moreover, in the same study, expression of an Akt-2 antisense mRNA inhibited tumorigenesis.

Little has been reported on Akt3 thus far. In one study, both Akt3 mRNA levels and enzymatic activity were elevated in breast cancer cell lines and tumors lacking the estrogen receptor as well as in prostate cancer cell lines that are androgen-insensitive. These results indicate that Akt 3 may contribute to the more aggressive phenotype of the hormone-refractory breast and prostate carcinomas (Nakatani et al., 1999).

Myr-Akt is targeted to the plasma membrane and thus constitutively active. Both, preferential location to the membrane and kinase activity are required for oncogenicity by Akt (Aoki, Batista, Bellacosa, Tschlis, & Vogt, 1998). All three Akts are equally and strongly transforming in chicken embryo fibroblasts (CEFs) and induce the formation of hemangiosarcomas in chicken wing xenograft assays (Mende, Malstrom, Tschlis, Vogt, & Aoki, 2001). The histology of these tumors is identical to the hemangiosarcomas Myr-p110 PI3K. These data suggest that each Akt family member might mediate transformation downstream of PTEN loss or PI3K activation.

A recent study using transgenic mice overexpressing both an activated mutant of Akt and a PI3K decoupled mutant of polyomavirus middle T antigen shows that Akt can contribute to tumor progression but does not restore the metastatic phenotype observed with the wild type middle T (Hutchinson et al., 2001). These results suggest that PI3K signaling can contribute to the metastatic phenotype via an Akt-independent mechanism as mentioned above.

#### 3.4. *Akt targets*

Translocations into the genes encoding Forkhead transcription factors have been described. Chromosomal translocations resulting in PAX3-FKHR (Fredericks et al., 1995) and PAX7-FKHR fusion proteins are a common occurrence in alveolar rhabdomyosarcomas (reviewed in (Barr, 2001). The fusion proteins contain

PAX3/PAX7 DNA binding domain and FKHR transactivation domain, are expressed at higher levels than the wild type counterparts, and are constitutively nuclear (del Peso, Gonzalez, Hernandez, Barr, & Nunez, 1999). The oncogenic activity of the fusion proteins is thought to be due at least in part to deregulation of PAX3/PAX7 target genes (Barr, 2001).

Similarly, chromosomal translocations involving the MLL gene with AFX (Borkhardt et al., 1997) and FKHL1 (Hillion, Le Coniat, Jonveaux, Berger, & Bernard, 1997) have been found in acute leukemias. It has been suggested that MLL-FKHL1 fusion protein may result not only in a MLL gain of function but also in a dominant negative effect on FKHL1 function (Ayton & Cleary, 2001). Indeed, both loss of growth inhibitory effect of AFX (Medema et al., 2000), and deregulation of FKHL1 apoptotic target genes (Brunet et al., 1999) can contribute to enhanced survival and tumorigenesis.

The notion that the three Forkhead transcription factors that are substrates of Akt are also targets of translocation in human cancer is intriguing and raises the possibility that the fusion proteins produced from these translocation may serve to interfere with normal Forkhead function.

Other Akt targets involved in cancer include p70<sup>S6K</sup> which is located at a site of amplification in breast cancer (Barlund et al., 2000) and eIF-4B amplification in head and neck squamous cell carcinomas (Franklin et al., 1999).

#### 4. THERAPEUTIC MANIPULATIONS

The PI3K/PTEN/Akt pathway may be readily amenable to pharmacological manipulations. The recent success in developing relatively selective kinase inhibitors such as Gleevec and Iressa, and the relatively limited side-effect profile attributed to these agents augers well for future drugs in this class. In this regard, the kinase components of the PI3K pathway are particularly exciting targets for the rational design of small molecules. An open question remains where along the pathway is one most likely to gain therapeutic advantage while minimizing toxicity. Inhibitors against receptor tyrosine kinases are clearly one way by which this pathway can be targeted, but will not be discussed here.

##### 4.1. PI3K

Wortmannin and LY294002 are molecules which disrupt the ATP binding pocket of PI3K and PI3K like enzymes. While wortmannin is an irreversible inhibitor, LY294002 is a competitive inhibitor. Both have been extensively used in cell culture studies and both induce growth inhibition at concentrations that inhibit class Ia PI3Ks. At higher concentrations they induce apoptosis (Yao & Cooper, 1995). For instance, ovarian cancer cells exhibiting activation of the PI3K/Akt pathway undergo apoptosis when treated with either wortmannin or LY294002 (Yuan et al., 2000). Moreover, in ovarian cancers implanted in the murine peritoneum, combinations of LY294002 and paclitaxel are more efficacious than paclitaxel alone (Hu, Hofmann, Lu, Mills, & Jaffe, 2002). Although extensively used in *in vitro*

studies neither wortmannin nor LY294002 have been translated to human cancer therapy thus far.

Despite the broad tissue distribution of PI3K isoforms, the evidence of functional specialization within class Ia kinases suggests that isoform-selective inhibitors may have an acceptable therapeutic index. P110 $\alpha$  is preferentially involved in insulin signaling whereas p110 $\beta$  is more likely to transmit mitogenic signals. p100 $\alpha$  and  $\beta$  are more restricted to the lymphocytic compartment and are attractive targets for the development of novel anti-inflammatory drugs (Stein & Waterfield, 2000). While p110 $\beta$  is perhaps the most attractive target, germline deletion in the mouse leads to embryonic lethality. Nonetheless this does not speak to the consequence of inhibition in the adult organism and experiments to temporally delete the catalytic and regulatory subunit genes of PI3K may help to delineate the consequence of PI3K loss to adult animals.

Small molecule inhibitors of PIKKs as potential anticancer drugs is a field of intense research. For example, LY294002-geldanamycin heterodimers have been synthesized with intent of selectively inhibit PI3K and PIKK family members (Chiosis, Rosen, & Sepp-Lorenzino, 2001). In addition, novel pyrrolo-quinoline derivatives exhibiting potent PIKK inhibition have been recently reported (Peng et al., 2002). For a further discussion on PI3K as target for drug development see Stein *et al.* (Stein & Waterfield, 2000).

#### 4.2. PTEN

Overexpression of PTEN in PTEN wild type cells has modest effects on cell signaling, proliferation or viability (Simpson & Parsons, 2001) suggesting that increasing the gene dosage of PTEN in normal cells may be well tolerated. On the other and, restoration of PTEN to PTEN-null cells results in either growth arrest, apoptosis, and inhibition of soft-agar and xenograft growth. Thus restoration of PTEN function to PTEN-null tumors is a possible strategy. Clearly, the rate-limiting step in this approach is the development of effective gene therapy vectors.

#### 4.3. Akt

Akt is an attractive target for the development of novel inhibitors that might prove beneficial in the treatment of cancers in which the PI3K/PTEN/Akt pathway is constitutively activated by any of the aforementioned upstream genetic events (e.g. receptor amplification, PI3KCA amplification, Akt amplification and PTEN deletion). The viability and relatively subtle phenotypes of the Akt1 and 2 knockout mice (Chen et al., 2001; Cho, Thorvaldsen, Chu, Feng, & Birnbaum, 2001) raise the possibility that there may be functional redundancy among these kinase, however, it is clear from these experiments that reduced levels of Akt activity can be tolerated during development and in adult mice. In addition, these data suggest that the Akt-1 and -2 may have evolved functional specifications. Thus, it will be critical to determine whether inhibition or genetic inactivation of specific Akt isoforms reverse the transformed phenotype associated with PTEN loss of PI3K activation. If differences in Akt isoforms are found in such studies then ideally, isoform-specific inhibitors could be generated to exploit such hypothetical differences. The

development of Akt3 knockout mouse as well as the disruption of Akt in a *pten*<sup>+/-</sup> background will provide further insights into the toxicity of inhibiting Akt activity *in vivo*.

Herbimacyn A and geldanamycin are ansamycin antibiotics that bind to and inhibit the heat-shock protein 90 (Hsp90) function. Hsp90 is a chaperone protein involved in the refolding of proteins during cellular stress and the conformational maturation of certain signaling proteins including Akt, HER2, Raf, EGFR and steroid receptors (Sausville, 2001). Hsp90 inhibition prevents refolding and leads to proteosomal degradation of those signaling molecules including significant reduction of Akt protein levels and a consequent downregulation of signaling through these pathways (Schneider et al., 1996). In addition, Hsp90 binds to and stabilizes the mature HER2 kinase domain. Thus, geldanamycin also stimulates HER2 degradation via disruption of the HER2/Hsp90 association (Xu et al., 2001).

17-allyl-aminogeldanamycin (17-AAG) is a geldanamycin derivative that has anti-tumor activity both in cell lines and xenograft assays. In breast cancer cells, 17-AAG causes RB-dependent G1 arrest and enhances the apoptotic effects of cytotoxic agents such as taxol in breast cancer cell (Munster, Basso, Solit, Norton, & Rosen, 2001). G1 arrest is associated with cyclin D loss and hypophosphorylation of RB.

In breast cancer cells with high levels of HER2, 17-AAG inhibits Akt in a complex manner. In addition to down-regulation of Akt expression, 17-AAG also causes a rapid inhibition of Akt kinase activity prior to proteosomal degradation of HER2 and Akt (Basso, Solit, Munster, & Rosen, 2002). 17-AAG is currently in clinical trials.

#### 4.4. FRAP/mTOR

As mentioned above, the genetic dissection of PI3K signalling in *Drosophila melanogaster* has linked PI3K signalling to the regulation of cell-size and proliferation to the *Drosophila* homologue of mTOR. Furthermore in PTEN-nulls cells there is elevated levels of phosphorylated 4EBP-1, a downstream translation effector of TOR signalling. These data have raised the possibility that rapamycin might have anti-tumor efficacy.

Rapamycin is a natural macrolide isolated from the microorganism *Streptomyces hygroscopicus*. It binds to the immunophilin FKBP12 and the drug-protein complex in turn binds with high affinity to mTOR. Rapamycin inhibits phosphorylation of mTOR targets p70<sup>S6K</sup> and 4E-BP resulting in decreased translation resulting in a G1 cell cycle arrest. Though principally a cytostatic drug, rapamycin can also induce cell death. Rapamycin inhibits immune cell proliferation and thus has been used clinically as an immunosuppressant.

The use of rapamycin has been limited by difficulties in solubility. Newer agents including CCI-779 and 40-O-(2-Hydroxyethyl)-rapamycin are esterified rapamycin derivatives with improved solubility and oral bioavailability. Recent data suggests that tumor lines or murine tumors lacking PTEN are particularly sensitive to CCI-779 (Neshat et al., 2001; Podsypanina et al., 2001). Moreover, CEFs that are directly transformed by Akt or PI3K are likewise sensitive to rapamycin when compared to

Ras transformed CEFs. These observations suggest that such tumors may depend on continuous TOR activity for either proliferation or survival. Finally, it has been recently reported that immunosuppressive doses of rapamycin can also inhibit tumor growth in mice probably by an anti-angiogenic effect linked to reduced production of vascular endothelial growth factor (VEGF) (Guba et al., 2002).

In human Phase I trials (dose escalation) treatment with CCI-779 has been associated with decreased platelet counts, diarrhea, vomiting, hypocalcemia and increase triglyceride levels but in general was well tolerated. Phase II trials are under way in glioblastoma multiforme, melanoma, prostate cancer, breast cancer and melanoma. Responses have been seen in a small number of tumors, however phase I trials are not primarily designed to assess efficacy and thus the Phase II data is eagerly awaited.

## 5. CONCLUSION

In conclusion, direct genetic alterations leading to deregulated PI3K/Akt signalling are common in a significant fraction of human malignancies. The forthcoming decade should witness the development and clinical deployment of a number of novel small molecule inhibitors specifically designed to disrupt the function of members of this pathway. It is hoped and perhaps likely that such inhibitors, either alone or in combination with current therapeutics, will ultimately prove clinically efficacious.

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